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Article

Induction of Innate Lymphoid Cell-Derived Interleukin-22 by the Transcription Factor STAT3 Mediates Protection against Intestinal Infection

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SUMMARY

Inhibitors of the transcription factor STAT3 target STAT3-dependent tumorigenesis but patients often develop diarrhea from unknown mechanisms. Here we showed that STAT3 deficiency increased morbidity and mortality after *Citrobacter rodentium* infection with decreased secretion of cytokines including IL-17 and IL-22 associated with the transcription factor RORγt. Administration of the cytokine IL-22 was sufficient to rescue STAT3-deficient mice from lethal infection. Although STAT3 was required for IL-22 production in both innate and adaptive arms, by using conditional gene-deficient mice, we observed that STAT3 expression in RORγt$^+$ innate lymphoid cells (ILC3s), but not T cells, was essential for the protection. However, STAT3 was required for RORγt expression in T helper cells, but not in ILC3s. Activated STAT3 could directly bind to the *Il22* locus. Thus, cancer therapies that utilize STAT3 inhibitors increase the risk for pathogen-mediated diarrhea through direct suppression of IL-22 from gut ILCs.

INTRODUCTION

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that regulates many genes involved in apoptosis, proliferation, migration, and survival in different cell types. STAT3 signaling is constitutively active in many types of tumor cells and tumor-associated immune cells during tumorigenesis, and this dysregulation promotes tumor growth and suppresses antitumor immune responses (Yu et al., 2007). Thus, STAT3 inhibitors have been explored in clinical trials for different cancer patients (Page et al., 2011). Sunitinib, an oral multitargeted tyrosine kinase inhibitor used for the treatment of several types of cancers including gastrointestinal tumors, also suppresses STAT3 activity in host immune cells (Xin et al., 2009). However, nearly half of the patients develop diarrhea after Sunitinib treatment with unclear pathogenesis (Schwandt et al., 2009). Mutations of *STAT3* have been shown to be related to hyper-immunoglobulin E syndrome patients with recurrent mucosal infections (Minegishi et al., 2007). Therefore, STAT3-related diarrhea could be linked with increased susceptibility to mucosal infections such as intestinal infections.

Citrobacter rodentium is a natural mouse extracellular enteric pathogen that mimics human enterohaemorrhagic *E. coli* and enteropathogenic *E. coli*, which are major causes of diarrheal disease worldwide that result in hundreds of thousands of deaths each year. Clearance of *C. rodentium* requires both the innate and adaptive immune responses (Bry and Brenner, 2004; Maaser et al., 2004). Both RORγt$^+$ group 3 innate lymphoid cells (ILC3s) and T helper (Th17, Th22) cells are important for the host to control *C. rodentium* infection (Basu et al., 2012; Ivanov et al., 2009; Qiu et al., 2012; Tumanov et al., 2011). Transferring either wild-type ILC3s cells (Sonnenberg et al., 2011) or Th22 cells (Basu et al., 2012) protects the mice from *C. rodentium* infection. However, it is not clear whether the innate or adaptive RORγt$^+$ lymphocytes are essential for protection against infection.

ILCs represent a family of immune cells that have morphological characteristics of lymphocytes but lack rearranged antigen receptors. ILCs can produce an array of effector cytokines that correspond to the cytokine profiles of the T helper cell subsets; for example, IFN-γ by group 1 ILCs (NK cells and ILC1) and Th1 cells, IL-5 and IL-13 by group 2 ILCs (ILC2) and Th2 cells, and IL-17 and IL-22 by ILC3s (including LTi, NCR$^+$ ILC3, and NCR$^-$ ILC3) and Th17 and Th22 cells (Spits et al., 2013; Spits and Cupedo, 2012; Spits and Di Santo, 2011). The current dogma is that development and function of innate and adaptive lymphoid cells are under the control of analogous transcription factors. T-bet is involved in the development of NK, ILC1, and Th1 cells (Fuchs et al., 2013; Gordon et al., 2012; Szabo et al., 2000), and GATA3 is critical for the development of both ILC2 and Th2 cells (Furusawa et al., 2013; Hoyler et al., 2012; Klein Wolterink et al., 2013; Mjösberg et al., 2012; Moro et al., 2010; Zheng and Flavell, 1997), whereas RORγt is required for the development of both ILC3s and Th17 cells (Eberl et al., 2004; Ivanov et al., 2006). STAT proteins are transcription factors involved in the differentiation of T helper cells (O'Shea et al., 2011). However, whether ILCs and T helper cells also share STAT protein for their development and downstream cytokines production is still unknown.

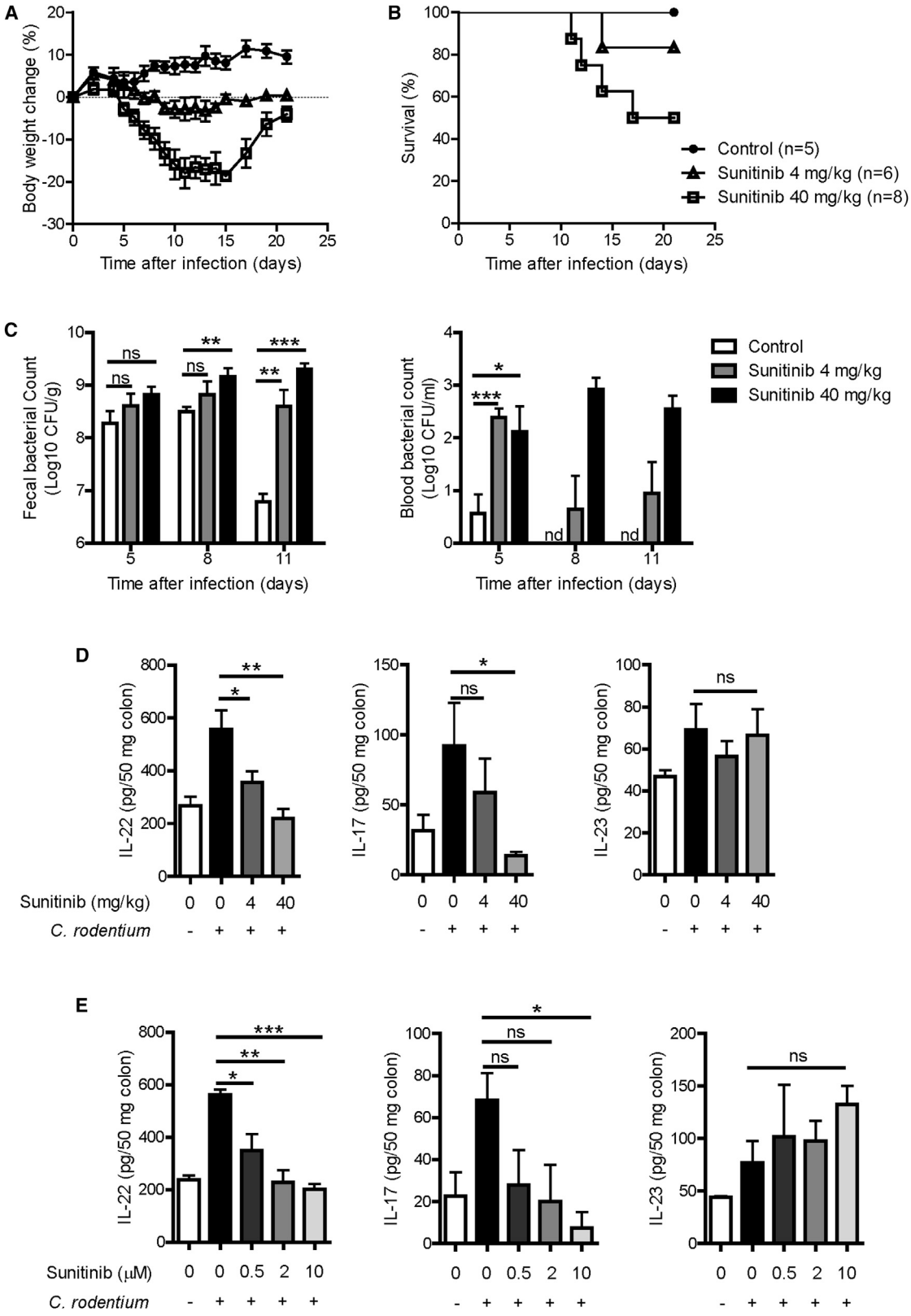

Figure 1. Sunitinib Impairs the Host Defense against Intestinal *C. rodentium* Infection

(A–C) 8-week-old wild-type mice were orally inoculated with 2 × 10^9 CFU of *C. rodentium*. Sunitinib (4 mg/kg, n = 6 or 40 mg/kg, n = 8) or control (n = 5) was administered orally from day 0 to day 7, once daily.

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Previous studies have shown that deletion of STAT3 in Th17 cells impairs their expression of RORγt and development (Ivanov et al., 2006; Laurence et al., 2007; O'Shea et al., 2011; Veldhoen et al., 2008; Yang et al., 2007, 2008; Zhou et al., 2007), but its role in ILCs has not been examined yet. Because innate and adaptive IL-17 producers share many transcriptional networks, it is expected that STAT3 also regulates RORγt expression in ILCs, analogous to their adaptive counterparts. However, in contrast to the STAT3-dependent development of Th17 and Th22 cells, we revealed that STAT3 does not control RORγt expression in ILCs, but rather directly regulates their production of IL-22 in response to infection.

RESULTS

Sunitinib Impairs the Host Defense against Intestinal *C. rodentium* Infection

To determine whether tyrosine kinase (including STAT3 pathway) inhibitor treatment increases the risk of gut infection, *C. rodentium*-infected mice were orally administered Sunitinib at clinically relevant doses (Xin et al., 2009). After *C. rodentium* infection, the mice with high-dose Sunitinib (40 mg/kg) treatment developed severe diarrhea and rapidly lost body weight, with nearly 50% mortality by 3 weeks; low-dose Sunitinib (4 mg/kg)-treated mice also developed diarrhea but lost less body weight, with few mortality by 3 weeks; however, neither weight loss nor death was observed in the control-treated mice (Figures 1A and 1B). Sunitinib treatment also resulted in increased bacterial titers in both feces and blood at days 8 and 11 postinfection (Figure 1C). Compared with control mice, the Sunitinib-treated mice exhibited severe colonic pathology with severe disruption of the epithelial layer and infiltration of inflammatory cells (Figure S1 available online). Together, these data indicate that oral Sunitinib administration could increase morbidity and mortality after intestinal *C. rodentium* infection.

Previous studies have shown that the Th17 and Th22 cell-related cytokines (IL-17, IL-22, and IL-23) are important for host defense against *C. rodentium* infection (Ishigame et al., 2009; Zheng et al., 2008). To determine whether the increased risk of intestinal infection by tyrosine kinase inhibition was due to impaired host immune responses, the colonic cytokine expression profile was examined at day 5 postinfection. In contrast to Th1 and Th2 cell-related cytokines, the Th17 and Th22 cell-related cytokines (IL-17 and IL-22) were upregulated after infection but were suppressed by both in vivo and in vitro Sunitinib treatment (Figures 1D and 1E and data not shown). Although IL-23 from DCs or macrophages is a major inducer for IL-22 production, Sunitinib had no effect on IL-23 production (Figures 1D and 1E). Taken together, these data indicate that Sunitinib impairs the host defense against intestinal *C. rodentium* infection. It raises the possibility that STAT3 might contribute to IL-22-dependent protection.

STAT3 Signaling from RORγt$^+$ Lymphocytes Controls *C. rodentium* Infection

IL-17 and IL-22 are known to be regulated by RORγt (encoded by *Rorc*) and are mainly produced by RORγt$^+$ ILCs and T cells (Ivanov et al., 2006; Qiu et al., 2012). To determine whether Sunitinib's adverse effect was due to STAT3 inhibition on ILC3s and T cells, *Stat3*-floxed mice were crossed with *Rorc-cre* transgenic mice to achieve specific deletion of STAT3 in RORγt-expressing cells (*Rorc-cre-Stat3*$^{fl/fl}$). Because RORγt is transiently expressed at high levels at the double-positive stage of T cell development, *Rorc-cre-Stat3*$^{fl/fl}$ mice lack STAT3 expression not only by RORγt$^+$ ILC3s, but also by most αβ T cells (Figures S2A–S2C).

We next tested whether STAT3 signaling is necessary for controlling the *C. rodentium* infection. After infection, *Rorc-cre-Stat3*$^{fl/fl}$ mice rapidly lost body weight and died around day 10, whereas no weight loss or death was observed in their littermate control wild-type (*Stat3*$^{fl/fl}$) mice (Figures 2A and 2B) or *Rorc-cre-Stat3*$^{fl/+}$ heterozygous mice (Figure S2D). To test whether the increased morbidity and mortality were associated with bacterial colitis, we checked bacterial titer and diarrhea status. *Rorc-cre-Stat3*$^{fl/fl}$ mice had 10–100 times higher bacterial titers in the feces compared to WT mice at days 5 and 8 postinfection (Figures 2C and S2E). *Rorc-cre-Stat3*$^{fl/fl}$ mice also showed increased bacterial titers in the blood, liver, and spleen (Figures 2D and S2E), suggesting systemic dissemination of *C. rodentium*. The *Rorc-cre-Stat3*$^{fl/fl}$ mice exhibited severe diarrhea, colonic shortening, severe inflammation, and tissue injury in the colon and severe disruption of the epithelial layer compared with WT mice (Figures 2E, 2F, and S2F). Together, these data indicate that STAT3 signaling in RORγt$^+$ cells is essential for host defense against a mucosal bacterial pathogen.

STAT3 Signaling from RORγt$^+$ Lymphocytes Controls Both IL-22 and IL-17 Production during *C. rodentium* Infection

We have shown that tyrosine kinase (including STAT3 pathway) inhibition suppressed both IL-22 and IL-17 expression after infection, which are required for the protection against *C. rodentium* infection (Ishigame et al., 2009; Ivanov et al., 2009; Wang et al., 2010; Zheng et al., 2008). We also measured IL-22 and IL-17 amounts in the colon of WT and *Rorc-cre-Stat3*$^{fl/fl}$ mice early after *C. rodentium* infection. Compared to WT mice, expression of IL-22 and IL-17 was significantly reduced in the colon of *Rorc-cre-Stat3*$^{fl/fl}$ mice on day 5 postinfection (Figure 3A). RegIIIγ and RegIIIβ, two antimicrobial proteins dependent on IL-22 (Pickert et al., 2009; Zheng et al., 2008), were also reduced in the colon of *Rorc-cre-Stat3*$^{fl/fl}$ mice after infection (Figure 3A). These results suggest that

(A and B) Body weight was measured at the indicated time points (A) and survival rates (B) are shown.
(C) Bacterial titers from blood and fecal homogenate cultures at indicated day postinfection in the mice in (A) and (B).
(D) IL-22, IL-17, and IL23 levels from the colon culture supernatants of Sunitinib- or control-treated mice at day 5 after infection (n = 5 each group).
(E) The colons from untreated mice (n = 3) were removed at day 5 after infection and cultured with Sunitinib at indicated concentrations for 24 hr in vitro. IL-22, IL-17, and IL-23 levels were examined in the colon culture supernatants.
*p < 0.05, **p < 0.01, ***p < 0.001; ns, no significant difference (Student's t test); nd, nondetectable. Data are representative of two independent experiments (mean ± SEM). See also Figure S1.

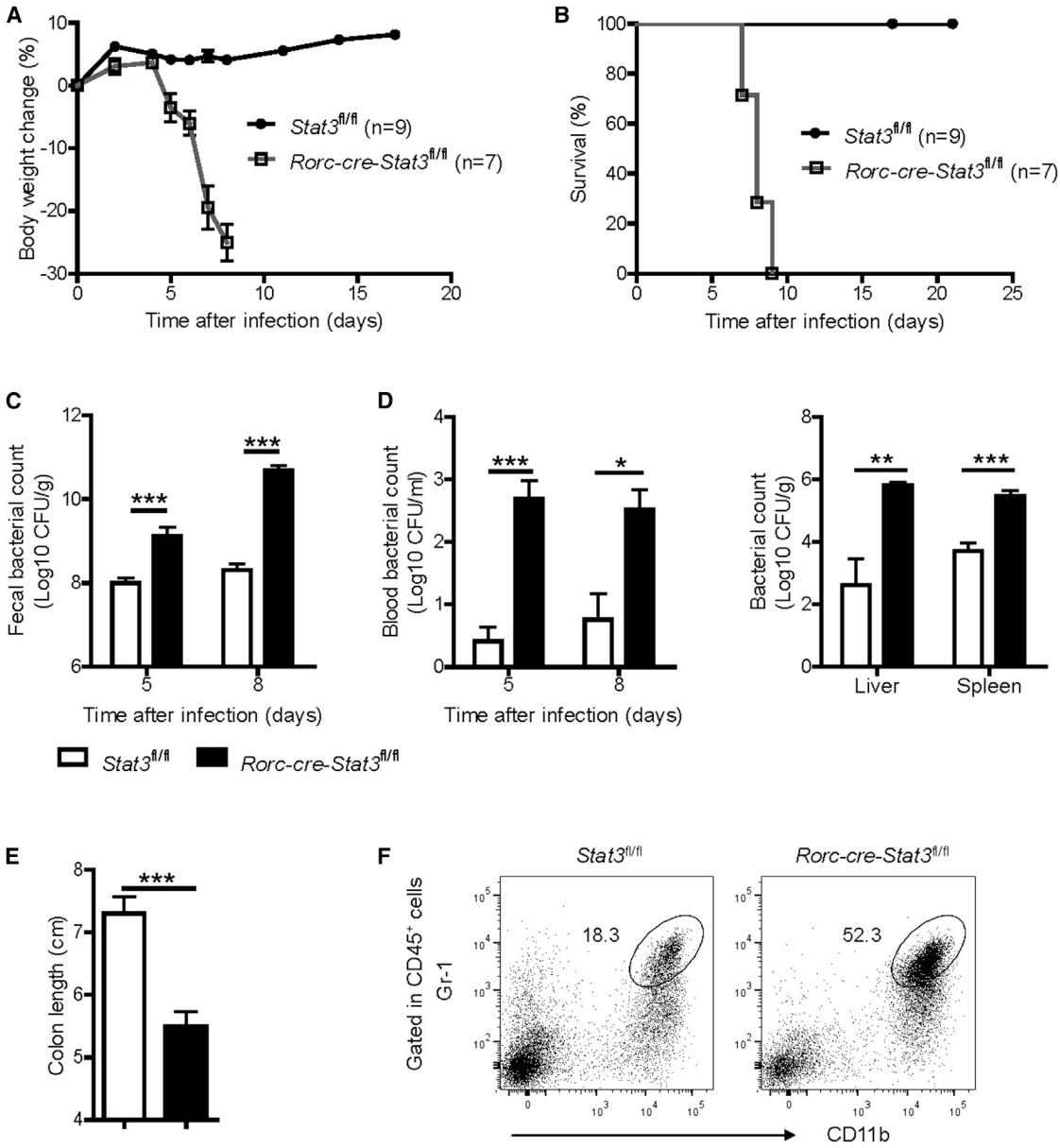

Figure 2. STAT3 Signaling Is Essential for Control of Intestinal *C. rodentium* Infection

7-week-old *Rorc-cre-Stat3*$^{fl/fl}$ (n = 7) and their littermate wild-type *Stat3*$^{fl/fl}$ (n = 9) mice were orally inoculated with *C. rodentium* (2×10^9 CFU) and body weight was measured at the indicated time points.

(A and B) Body weight change (A) and survival rates (B) are shown.

(C) Bacterial titers from fecal homogenate cultures at indicated day postinfection in the mice in (A) and (B).

(D) Bacterial titers from blood and spleen, liver homogenate cultures at indicated day postinfection are shown.

(E) *Rorc-cre-Stat3*$^{fl/fl}$ mice show colon shortening at day 8 postinfection.

(F) *Rorc-cre-Stat3*$^{fl/fl}$ mice show more inflammatory monocyte or neutrophil infiltration in the colon. LPLs were isolated from colon and gated in CD45$^+$ cells.

$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's t test). Data are representative of three independent experiments (A–C; mean ± SEM) or two independent experiments (D, E; mean ± SEM). See also Figure S2.

STAT3 signaling is essential for IL-22 and IL-17 production in the gut after mucosal bacterial infection.

Exogenous IL-22 but Not IL-17 Rescues STAT3-Deficient Mice during *C. rodentium* Infection

Because both IL-17 and IL-22 are controlled by STAT3 signaling, we wondered whether administration of exogenous IL-17 or IL-22 is sufficient to rescue *Rorc-cre-Stat3*$^{fl/fl}$ mice from death by *C. rodentium* infection. To address this question, IL-17A- and IL-22-expressing plasmids were hydrodynamically injected into *Rorc-cre-Stat3*$^{fl/fl}$ mice on the same day as *C. rodentium* infection. All of the *Rorc-cre-Stat3*$^{fl/fl}$ mice treated with IL-22 survived after infection and lost weight only slightly, whereas vector and IL-17-alone-treated mice rapidly lost body weight and

succumbed to *C. rodentium* infection (Figures 3B and 3C). Accordingly, IL-22-treated *Rorc-cre-Stat3*$^{fl/fl}$ mice had reduced bacterial counts in their feces and blood compared to vector and IL-17-alone-treated mice (Figure 3D). Collectively, these data demonstrate that exogenous IL-22 but not IL-17 is able to rescue *Rorc-cre-Stat3*$^{fl/fl}$ mice from a lethal *C. rodentium* infection and that IL-22 is an essential and sufficient pathway downstream of STAT3 signaling to protect mice against mucosal bacterial pathogens.

STAT3 Signaling Is Required for Both Innate and Adaptive IL-22 Production

Previous studies have shown that *C. rodentium* infection induces an early wave of IL-22 production by ILC3s and a second wave by CD4$^+$ T (Th22) cells (Basu et al., 2012; Qiu et al., 2012; Tumanov et al., 2011). To test whether STAT3 controls innate or adaptive IL-22 production, we examined cytokine expression in the intestinal lamina propria lymphocytes (LPLs) isolated from both naive and infected *Rorc-cre-Stat3*$^{fl/fl}$ and wild-type mice by intracellular cytokine staining. Consistent with the previous studies (Basu et al., 2012; Qiu et al., 2012), CD3$^-$ innate LPLs were the main IL-22 producers compared to CD3$^+$ T cells in naive and infected WT mice at an early stage of infection (day 4), although there were more IL-22-producing T cells than ILCs at a later stage of infection (day 8) in WT mice (Figure 3E). However, *Rorc-cre-Stat3*$^{fl/fl}$ mice had severely decreased expression of IL-22 by intestinal LPLs, regardless of CD3$^-$ innate cells or CD3$^+$ T cells (Figures 3E, S3A, and S3B). Moreover, Sunitinib treatment also suppressed IL-22 production in innate LPLs from wild-type mice (Figure S3E). In a similar manner as IL-22, the IL-17 production was also significantly reduced in both innate and adaptive lymphocytes (Figures S3C and S3D). Because PMA and ionomycin are not physiological stimuli, the LPLs were also stimulated with anti-CD3 and anti-CD28 to mimic TCR signaling. Similar to PMA and ionomycin treatment, STAT3-deficient T cells also showed little IL-22 and IL-17 production after anti-CD3 and anti-CD28 stimulation (Figure S3F). Together, our data indicate that STAT3 signaling is essential for IL-22 and IL-17 expression in both ILC3s and T cells.

STAT3 Signaling and IL-22 from ILC3s but Not from T Cells Are Essential for the Control of *C. rodentium* Infection

Because STAT3 signaling regulates IL-22 expression in both innate and adaptive lymphocytes, we wondered whether innate or adaptive IL-22 is more important for the protection from *C. rodentium* infection. To test whether the STAT3 signaling from ILC3s is sufficient for the protection against *C. rodentium* infection in the transient absence of T cells, CD4 mAb was administered to infected *Rorc-cre-Stat3*$^{fl/fl}$ and WT mice to deplete the CD4$^+$ T cells. To avoid depletion of CD4$^+$ LTi cells, we used a low dose of CD4 mAb to transiently remove CD4$^+$ T cells but not CD4$^+$ ILC3s. As shown in Figure S4A, 90% of peripheral CD4$^+$ T cells were depleted, but there was no proportional reduction of ILC3s in the lamina propria after multiple CD4 mAb injections. Because CD4$^+$ T cells are required for host adaptive immunity especially for antibody responses against *C. rodentium* (Bry et al., 2006), depletion of CD4$^+$ T cells provided another model to study whether the innate immune response is sufficient against this pathogen in the absence of adaptive responses. Treatment with CD4 mAb resulted in reduction of *C. rodentium*-specific IgG in WT mice and all of them died at 3 weeks after *C. rodentium* infection (Figures S4B, 4A, and 4B), which is the same as *Rag1*$^{-/-}$ mice, suggesting that adaptive immunity is responsible for the late phase of protection. Treatment of *Rorc-cre-Stat3*$^{fl/fl}$ mice with additional CD4 mAb did not aggravate or ameliorate the *C. rodentium* infection compared with rat IgG (control for CD4 mAb) treatment group. All of the *Rorc-cre-Stat3*$^{fl/fl}$ mice with or without depletion of CD4$^+$ T cells still died on day 8 postinfection (Figures 4A–4C). These data suggest that STAT3 signaling and IL-22 from CD4$^+$ T cells are not essential for protecting mice in the early phase of *C. rodentium* infection.

To further test whether STAT3 signaling in T cells is required for the later T and B cell response and prevention of chronic infection, we rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice by exogenous IL-22 and examined the antibody production. IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice produced the same amount of *C. rodentium*-specific IgG as did WT mice (Figure S4C). Moreover, 60 days after first *C. rodentium* infection, IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice were challenged with a high dose of *C. rodentium* again. Interestingly, none of the IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice showed diarrhea (data not shown) or weight loss (Figure S4D), suggesting that IL-22-rescued *Rorc-cre-Stat3*$^{fl/fl}$ mice developed normal protection. Together, these data demonstrate that STAT3 signaling from CD4$^+$ T cells is not essential for normal IgG production and protecting mice after *C. rodentium* infection.

To further test whether the STAT3 signaling from RORγt$^+$ ILCs is sufficient for the protection against *C. rodentium* infection, we crossed the *Stat3*-floxed mice with *Cd4-cre* mice to generate mice lacking STAT3 only in T cells (*Cd4-cre-Stat3*$^{fl/fl}$) but not in CD4$^+$RORγt$^+$ ILCs (Eberl and Littman, 2004) (data not shown). Even after a high dose of *C. rodentium* infection, the *Cd4-cre-Stat3*$^{fl/fl}$ mice did not lose body weight and all survived (Figure 4D). Furthermore, the fecal titers of *C. rodentium* were not significant higher in *Cd4-cre-Stat3*$^{fl/fl}$ mice than in WT mice 5–11 days after infection (Figure 4E). Similar to the *Rorc-cre-Stat3*$^{fl/fl}$ mice, *Cd4-cre-Stat3*$^{fl/fl}$ mice also lacked IL-22-producing CD4$^+$ T cells in the gut (Figures 4F and 4G). However, the IL-22-producing ILCs in *Cd4-cre-Stat3*$^{fl/fl}$ mice were not reduced compared with WT mice, which were different with *Rorc-cre-Stat3*$^{fl/fl}$ mice (Figures 3E, 4F, and 4G). Furthermore, CD4 mAb was also administered to *Cd4-cre-Stat3*$^{fl/fl}$ mice and *Stat3*$^{fl/fl}$ mice. As shown in Figures S4E and S4F, both CD4 mAb-treated *Stat3*$^{fl/fl}$ and *Cd4-cre-Stat3*$^{fl/fl}$ mice lost body weight and died around day 20 postinfection, whereas untreated mice survived, suggesting that CD4$^+$ T cells are important for the protection against *C. rodentium* infection but that STAT3 signaling from T cells is not essential. Together, these data demonstrate that STAT3 signaling and IL-22 from ILC3s, but not CD4$^+$ T cells, are essential for protecting mice from death during *C. rodentium* infection.

CD90^{hi}CD45^{lo} ILC3s Protect *Rorc-cre-Stat3*$^{fl/fl}$ Mice during *C. rodentium* Infection

Because we have shown that STAT3 signaling from ILC3s but not T cells is essential for the protection from *C. rodentium* infection, we wondered whether adoptive transfer of ILC3s could

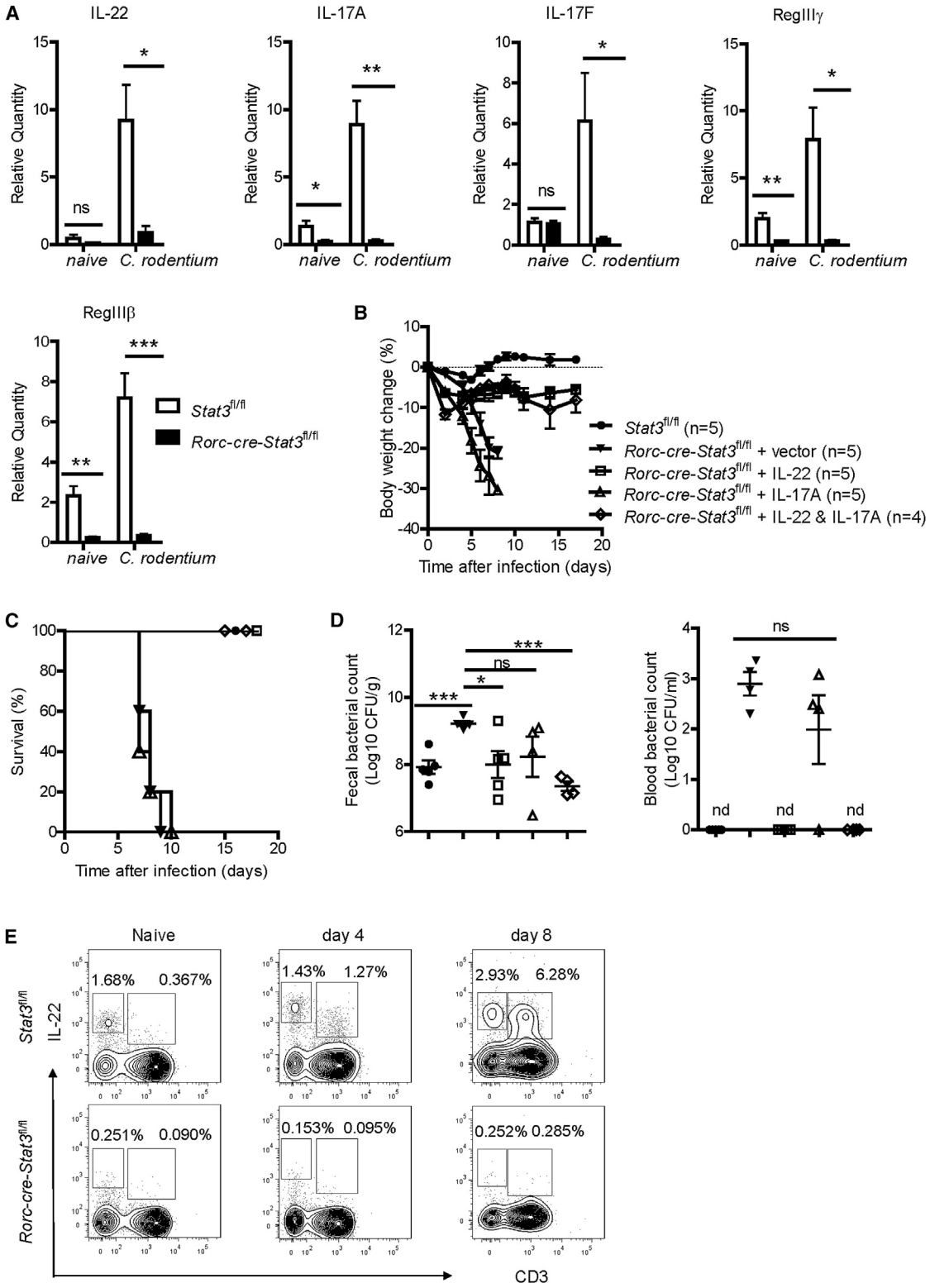

Figure 3. IL-22, but Not IL-17, Is an Essential and Sufficient Pathway Downstream of STAT3 Signaling to Protect Mice against *C. rodentium* Infection

(A) *Rorc-cre-Stat3*$^{fl/fl}$ and wild-type *Stat3*$^{fl/fl}$ littermate mice (n = 5 per group) were infected with 2 × 10^9 CFU of *C. rodentium*. The mRNA expression of IL-22, IL-17A, IL-17F, and IL-22-dependent RegIIIγ and RegIIIβ antimicrobial proteins in the colon of naive or 5 days infected mice were measured by real-time PCR. *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t test). Data are representative of two independent experiments (mean ± SEM).

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rescue *Rorc-cre-Stat3*$^{fl/fl}$ mice from infection. However, because of extremely low number of ILC3s and limited *Rorc*$^{gfp/+}$ mice, it was difficult to get enough ILC3s. To find a better cell surface marker to identify ILC3s, we screened a large panel of surface markers and found that ILC3s could be strictly identified by CD45 and CD90. As shown in Figure 5A, after gating with CD45 and CD90, CD90$^+$ LPLs from the large intestine and small intestine of *Rag1*$^{-/-}$ mice fell into three separate populations: CD90^{hi}CD45^{lo}, CD90^{lo}CD45^{int}, and CD90^{lo}CD45^{hi}. Most of CD90^{hi}CD45^{lo} LPLs (96%) were RORγt$^+$ ILC3s, and most of CD90^{lo}CD45^{int} LPLs (95%) were NK1.1$^-$RORγt$^-$ ILCs, whereas nearly three fourths of CD90^{lo}CD45^{hi} LPLs were NK1.1$^+$ NK cells. Similar to *Rag1*$^{-/-}$ mice, CD90$^+$ LPLs from both large intestine and small intestine of C57BL/6 WT mice fell into two separate populations: CD90^{hi}CD45^{lo} and CD90^{lo}CD45^{hi}. Most of CD90^{hi}CD45^{lo} LPLs (94%) were RORγt$^+$ ILC3s, whereas CD90^{lo}CD45^{hi} LPLs were CD3$^+$ T cells and other RORγt$^-$ ILCs (Figure S5). These data suggest that CD90^{hi}CD45^{lo} LPLs are specific cell surface markers for intestinal RORγt$^+$ ILC3s.

Then we sorted CD90^{hi}CD45^{lo} ILC3s and transferred small number of them into *Rorc-cre-Stat3*$^{fl/fl}$ mice with *C. rodentium* infection. Compared with untreated *Rorc-cre-Stat3*$^{fl/fl}$ mice, ILC3s-transferred *Rorc-cre-Stat3*$^{fl/fl}$ mice lost less body weight and about one third of the mice survived and recovered from the *C. rodentium* infection (Figures 5B and 5C). Accordingly, ILC3s-transferred *Rorc-cre-Stat3*$^{fl/fl}$ mice exhibited reduced bacterial counts in their feces and blood compared to untreated mice (Figure 5D). Collectively, these data demonstrate that CD90^{hi}CD45^{lo} ILC3s are sufficient to protect mice against *C. rodentium* infection.

The Development of RORγt$^+$ Th Cells, but Not RORγt$^+$ ILC3s, Requires STAT3 Signaling

Because *Rorc-cre-Stat3*$^{fl/fl}$ mice showed reduced IL-22 in both ILCs and T cells, we next determined how STAT3 regulates IL-22 production. IL-22 is mainly produced in RORγt$^+$ cells, and RORγt directly regulates IL-22 production at the transcriptional level. Previous studies have shown that STAT3 regulates the development of RORγt$^+$ Th17 cells (Ivanov et al., 2006; Laurence et al., 2007; Yang et al., 2007). To determine whether STAT3 signaling is shared by both innate and adaptive colonic RORγt$^+$ cells for their development, we examined the RORγt expression in the intestinal LPLs isolated from *Rorc-cre-Stat3*$^{fl/fl}$ mice and their littermate control wild-type mice. Consistent with *Cd4-cre-Stat3*$^{fl/fl}$ mice (Figure S6A), both the percentage and number of RORγt$^+$ Th cells (CD3$^+$CD4$^+$RORγt$^+$) in the colon were much lower in *Rorc-cre-Stat3*$^{fl/fl}$ mice compared with the WT mice (Figures 6A–6C and S6B). Unexpectedly, there were no differences in the percentages and numbers of the total RORγt$^+$ ILC3s (Figures 6D–6F), the subsets of ILC3s (LTi, NCR$^+$ ILC3, and NCR$^-$ ILC3; Figures 6D and S6C), or other ILCs (ILC1, ILC2; Figure S6C) between *Rorc-cre-Stat3*$^{fl/fl}$ and WT mice, suggesting that STAT3 was required for RORγt expression in T cells but not ILC3s or maintenance of ILC3s. Moreover, similar to naive mice, there were no differences in percentages and total numbers of the ILC3s after *C. rodentium* infection between *Rorc-cre-Stat3*$^{fl/fl}$ and WT mice (Figures 6E and 6F). Taken together, our data indicate that STAT3 signaling is essential for the development of RORγt$^+$ Th cells, but not ILC3s, and that STAT3-controlled IL-22 production is not through regulating RORγt expression in innate cells, which also suggests that RORγt is not sufficient to induce IL-22 production in the absence of STAT3 signaling.

STAT3 Directly Binds to the *Il22* Locus and Regulates IL-22 Production

Previous studies have shown that IL-23 is an inducer of IL-22 (Zheng et al., 2008). IL-23 interacts with the IL-23 receptor (IL-23R) and activates STAT3, which has multiple target genes, such as *Il23r*, *Rorc*, and *Ahr* in T cells (Ghoreschi et al., 2010; Ivanov et al., 2006; Veldhoen et al., 2008). RORγt and Ahr can bind to the *Il22* locus and directly promote IL-22 production (Qiu et al., 2012). Because the expression of RORγt in ILCs was not reduced in *Rorc-cre-Stat3*$^{fl/fl}$ mice, we began to explore other pathways, such as IL-23R or Ahr, defects in which could result in a defective response to IL-23 and subsequently reduced expression of IL-22. To test this hypothesis, we first examined the expression of these related genes in the colon of WT and *Rorc-cre-Stat3*$^{fl/fl}$ mice before or early after *C. rodentium* infection. Unexpectedly, there were no significant reductions of IL-23p19, p40, IL-23R, and Ahr in the *Rorc-cre-Stat3*$^{fl/fl}$ mice compared with WT mice, and the expression of RORγt was actually increased in the STAT3-deficient mice, probably as a secondary response to the increased infection (Figure 7A). Interestingly, IL-6 was increased in *Rorc-cre-Stat3*$^{fl/fl}$ mice after infection, indicating increased inflammation. Even with higher expression of RORγt, the IL-22 production was still impaired, suggesting that the regulation of IL-22 production by STAT3 signaling may not be mediated through these related pathways.

To further study the mechanism of how STAT3 regulates IL-22, ILC3s were purified from both *Rorc*$^{gfp/+}$ and *Rorc-cre-Stat3*$^{fl/fl}$ *Rorc*$^{gfp/+}$ mice by flow cytometric sorting and stimulated with IL-23. Consistent with the previous results, STAT3 is required for IL-22 production in ILC3s after IL-23 stimulation. However, there were no significant differences in IL-23R, RORγt, and Ahr between STAT3-deficient and WT ILC3s (Figure 7B). Together, these data suggest that STAT3 regulation of IL-22 is not through IL-23R, RORγt, or Ahr.

(B–D) *Stat3*$^{fl/fl}$ (n = 5) and *Rorc-cre-Stat3*$^{fl/fl}$ mice were orally inoculated with 2 × 10^9 CFU of *C. rodentium*. 10 μg control vector (n = 5), IL-17- (n = 5), IL-22- (n = 5), or both IL-17 and IL-22- (n = 4) expressing plasmids were hydrodynamically injected into *Rorc-cre-Stat3*$^{fl/fl}$ mice through the tail vein 6 hr after *C. rodentium* infection.
(B and C) Body weight changes (B) and survival rates (C) were monitored at indicated time points.
(D) CFU counts of *C. rodentium* in the fecal pellets and blood on day 5 after infection are shown. Data are representative of two independent experiments (mean ± SEM). *p < 0.05, ***p < 0.001; ns, no significant difference (Student's t test); nd, nondetectable.
(E) STAT3 signaling is required for both innate and adaptive IL-22 production. IL-22 expression in CD3$^-$ and CD3$^+$ cells were analyzed by intracellular cytokine staining followed by flow cytometry. *Rorc-cre-Stat3*$^{fl/fl}$ and wild-type *Stat3*$^{fl/fl}$ littermate mice were infected with 2 × 10^9 CFU of *C. rodentium*. Intestinal LPLs were isolated from the colons of *Stat3*$^{fl/fl}$ or *Rorc-cre-Stat3*$^{fl/fl}$ mice at day 4 and day 8 postinfection. Cells were stimulated with IL-23 (25 ng/ml), PMA (50 ng/ml), and ionomycin (750 ng/ml) for 4 hr and gated in Thy1$^+$ lymphocytes. Naive mice were used as control. Data are representative of three independent experiments. See also Figure S3.

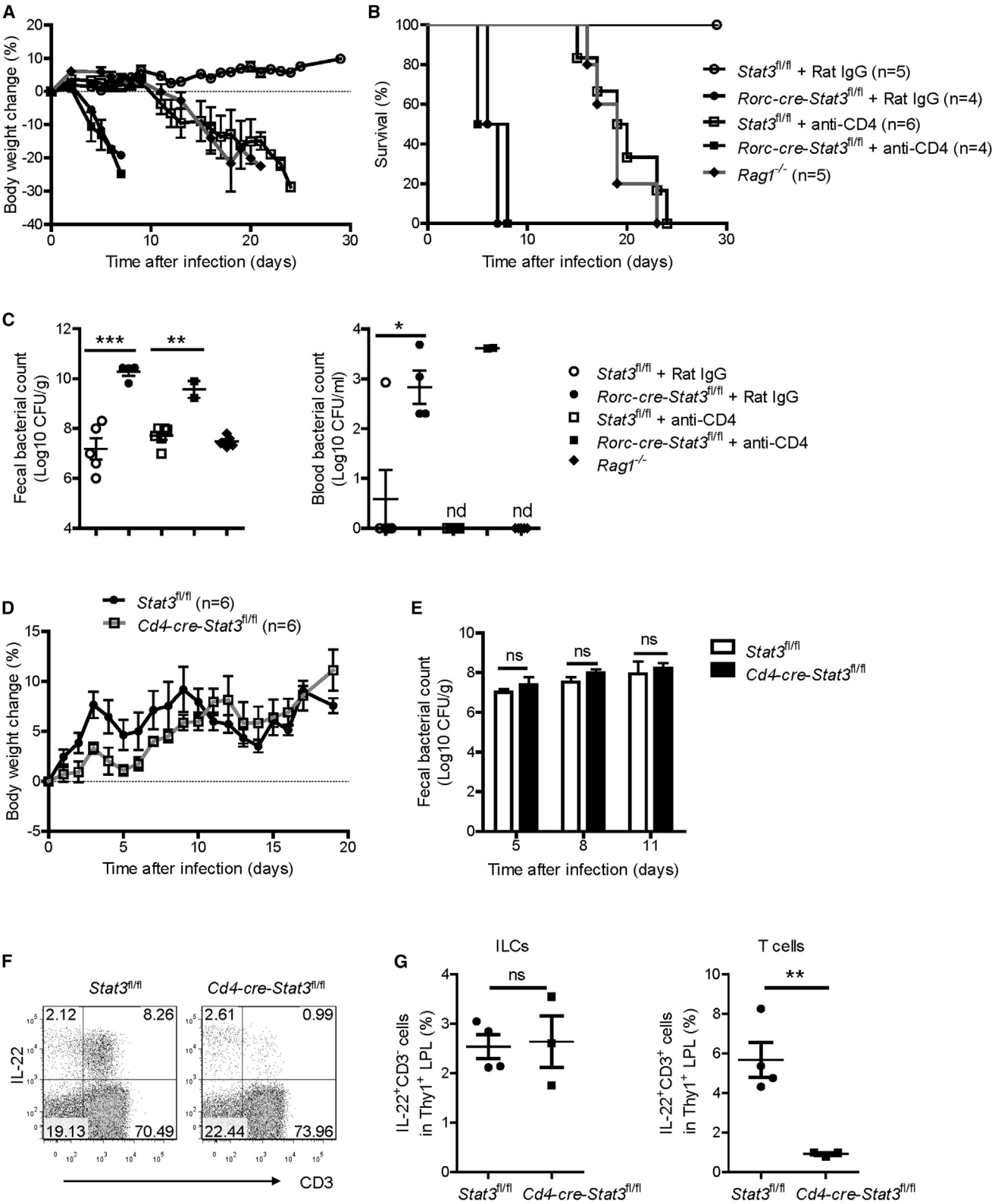

Figure 4. STAT3 Signaling and IL-22 from RORγt⁺ ILCs, but Not T Cells, Are Essential for Protection from *C. rodentium* Infection

(A–C) *Rorc-cre-Stat3*$^{fl/fl}$ and wild-type *Stat3*$^{fl/fl}$ mice were infected with 5 × 10^6 CFU of *C. rodentium* and injected intraperitoneally with mAb GK1.5 or rat IgG (50 μg per mouse each time) at day −5, 0, 5, 10, and 15 postinfection for depletion of CD4$^+$ T cells (4 to 6 mice per group).

(A and B) Average body weight change (A) and survival rates (B) at the indicated time points are shown.

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STAT3 is a transcription factor that directly regulates IL-17 production (Yang et al., 2011). Therefore, we wondered whether STAT3 can also directly regulate IL-22 production at the transcriptional level. By using an in silico approach, we found multiple STAT3 binding sites at the promoter and intron 1 of the *Il22* gene (Hutchins et al., 2012), suggesting that the *Il22* locus may be a direct target of STAT3 (Figure 7C). Indeed, by using EL4 lymphoma cells stably expressing the double flag peptide (DTFC) and double flag-tagged STAT3C (DFTC-STAT3C), an active form of STAT3 (Zhou et al., 2007), a chromatin immunoprecipitation (ChIP) assay by anti-flag antibodies showed enhanced recruitment of STAT3 to the *Il22* locus in cell lines stably expressing DFTC-STAT3C, suggesting that *Il22* is a direct target gene of STAT3 (Figure 7D). To further confirm binding of STAT3 to the *Il22* locus in primary cells, we sorted ILC3s from the intestinal LPLs of *Rag1*$^{-/-}$ mice. ChIP assay with anti-STAT3 antibody also showed enhanced recruitment of STAT3 to the *Il22* locus in ILC3s (Figure 7E). Together, these data suggest that STAT3 could directly bind to the *Il22* locus and promote IL-22 production in RORγt$^+$ cells.

DISCUSSION

IL-22-producing innate lymphoid cells were shown to control the dissemination of commensal bacteria and prevent systemic inflammation in naive mice, which correlates with chronic infectious diseases in humans, particularly in patients undergoing prolonged treatments that increase their risk for nosocomial infections of bacterial origins (Sonnenberg et al., 2012). However, how IL-22 is regulated by various transcriptional factors is not fully understood. Our results show that the production of IL-22 in both innate and adaptive lymphoid cells is dependent on STAT3, suggesting that STAT3 inhibition or deficiency may not only increase the chance of pathogen infection in the gut but also result in the disruption of normal gut homeostasis.

Th22 cells are an important source of IL-22 for host protection against low-dose *C. rodentium* infection (Basu et al., 2012). Here, we carefully utilized a complementary collection of conditional gene-deficient mice combined with T cell-specific depletion to show that IL-22 from T cells is not necessary for conferring protection even after high doses of *C. rodentium* inoculation. Although adoptive transfer of IL-22-producing T cells rescues IL-22-deficient mice from lethal infection (Basu et al., 2012), this suggests that additional IL-22-producing T cells can sufficiently control infection but does not prove that Th22 cells are essential for the infection. Consistently, exogenous IL-22 can sufficiently rescue many different gene-deficient mice from *C. rodentium* infection (Ota et al., 2011; Qiu et al., 2012; Tumanov et al., 2011; Zheng et al., 2008). Our data suggest that STAT3 and IL-22 from ILCs, but not Th22 cells, are essential for protecting the host in normal physiology state.

It is thought that the development and function of ILCs and Th cells are under the control of corresponding transcription factors (Hoyler et al., 2012; Zhou, 2012). However, our data suggest that the transcriptional regulation of ILC3s and Th17 and Th22 cells is complex and may be distinct between innate and adaptive lymphocytes. In contrast to the essential role of controlling RORγt expression in T cells (Yang et al., 2007; Zhou et al., 2007), STAT3 has little impact on the development of RORγt$^+$ ILC3s. This finding could explain the major differences in ontogeny between RORγt$^+$ Th cells and ILC3s. ILC3s first appear as fetal LTi cells that are generated on E12.5 during ontogeny in the liver, whereas RORγt$^+$ Th17 and Th22 cells are differentiated from naive CD4$^+$ T cells after birth dependent on the commensal microbiota (Eberl, 2012). Germ-free mice mostly lack RORγt$^+$ Th cells. Colonization of germ-free mice with segmented filamentous bacterium is sufficient to induce Th17 cells in the small intestine (Ivanov et al., 2009). In contrast, the development of ILC3s is independent of gut flora, although there are debates about the influence of symbiotic microbiota on some subsets or function of ILC3s (Sanos et al., 2009; Satoh-Takayama et al., 2008; Sawa et al., 2011; Sonnenberg and Artis, 2012). Our data suggest that STAT3 signaling is necessary for the host to sense the environmental microbiota and subsequently generate RORγt$^+$ Th cells but not to "program" the expression of RORγt in microbiota-independent ILCs. Thus, the mechanisms underlying the differential regulation RORγt expression by STAT3 in ILCs and T cells remain to be defined.

STAT3 is one of the downstream adaptors of IL-23R signaling and regulates the expression of RORγt, Ahr, and IL-23R in T cells (Ghoreschi et al., 2010; Ivanov et al., 2006; Veldhoen et al., 2008). Presumably, IL-23 activates the STAT3 signaling, which induces RORγt, Ahr, and IL-23R expression, and then subsequent induction of RORγt and Ahr regulates IL-22 production. However, our data show that the STAT3 deficiency in ILC3s has minimal impact on the expression of RORγt, Ahr, and IL-23R, but rather activated STAT3 directly binds to the *Il22* locus to regulate IL-22 transcription, suggesting that the expression of RORγt in ILC3s is not sufficient to promote IL-22 production in the absence of STAT3. Both RORγt and STAT3 are required for IL-22 production, and RORγt regulating IL-22 production is dependent on STAT3 signaling in ILCs, raising the questions of whether RORγt controls STAT3 signaling and how STAT3 and RORγt cooperate on regulating IL-22 production in ILCs.

(C) Bacterial titers from blood and fecal homogenate cultures at day 5 postinfection. Each dot represents one individual mouse.
(D and E) *Cd4-cre-Stat3*$^{fl/fl}$ (n = 6) and wild-type *Stat3*$^{fl/fl}$ (n = 6) mice were orally infected with 2 × 10^9 CFU of *C. rodentium*.
(D) Body weight changes are shown at the indicated time points.
(E) Bacterial titers from fecal homogenate cultures at indicated day postinfection in the mice in (D).
(F and G) IL-22 expression in CD3$^-$ and CD3$^+$ cells were analyzed by intracellular cytokine staining followed by flow cytometry. *Cd4-cre-Stat3*$^{fl/fl}$ and wild-type *Stat3*$^{fl/fl}$ littermate mice were infected with 2 × 10^9 CFU of *C. rodentium*. LPLs were isolated from the colons at day 8 postinfection. Cells were stimulated with IL-23 (25 ng/ml), PMA (50 ng/ml), and ionomycin (750 ng/ml) for 4 hr and gated in Thy1$^+$ lymphocytes. Percentage of IL-22$^+$CD3$^-$ and IL-22$^+$CD3$^+$ cells in the Thy1+ LPLs are shown (G). Each dot represents one individual mouse.
*p < 0.05, **p < 0.01, ***p < 0.001; ns, no significant difference (Student's t test); nd, nondetectable. Data are representative of two independent experiments (mean ± SEM). See also Figure S4.

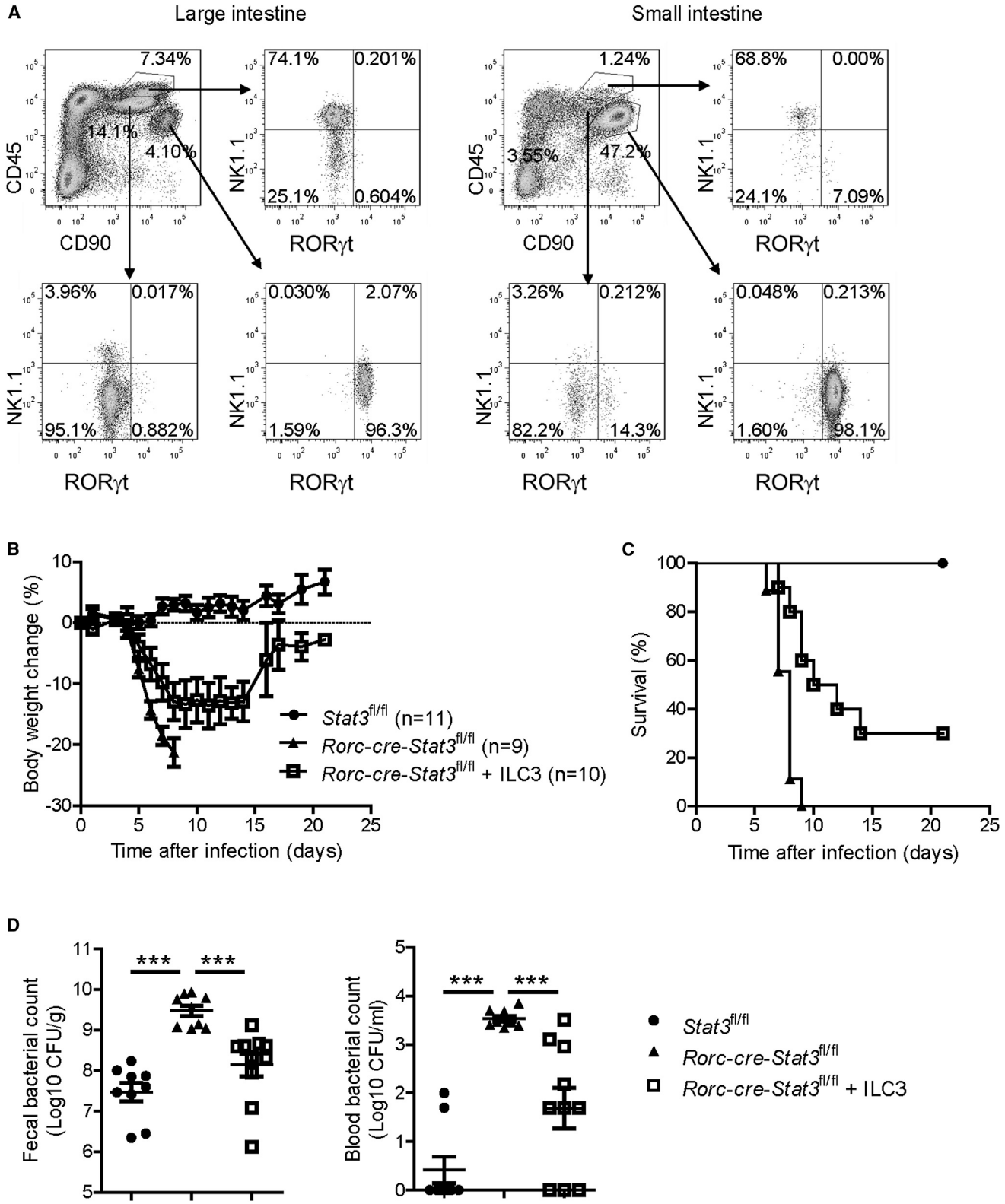

Figure 5. CD90^{hi}CD45^{lo} ILC3s Protect *Rorc-cre-Stat3*$^{fl/fl}$ Mice from *C. rodentium* Infection

(A) Intestinal CD90^{hi}CD45^{lo} LPLs are RORγt^{+} ILCs. LPLs were isolated from both the large intestine and small intestine of *Rag1*$^{-/-}$ mice and gated by CD45 and CD90 expression. Three separate populations, CD90^{hi}CD45^{lo}, CD90^{lo}CD45^{int}, and CD90^{lo}CD45^{hi} were further analyzed with the expression of NK1.1 and RORγt. Data are representative of two independent experiments.

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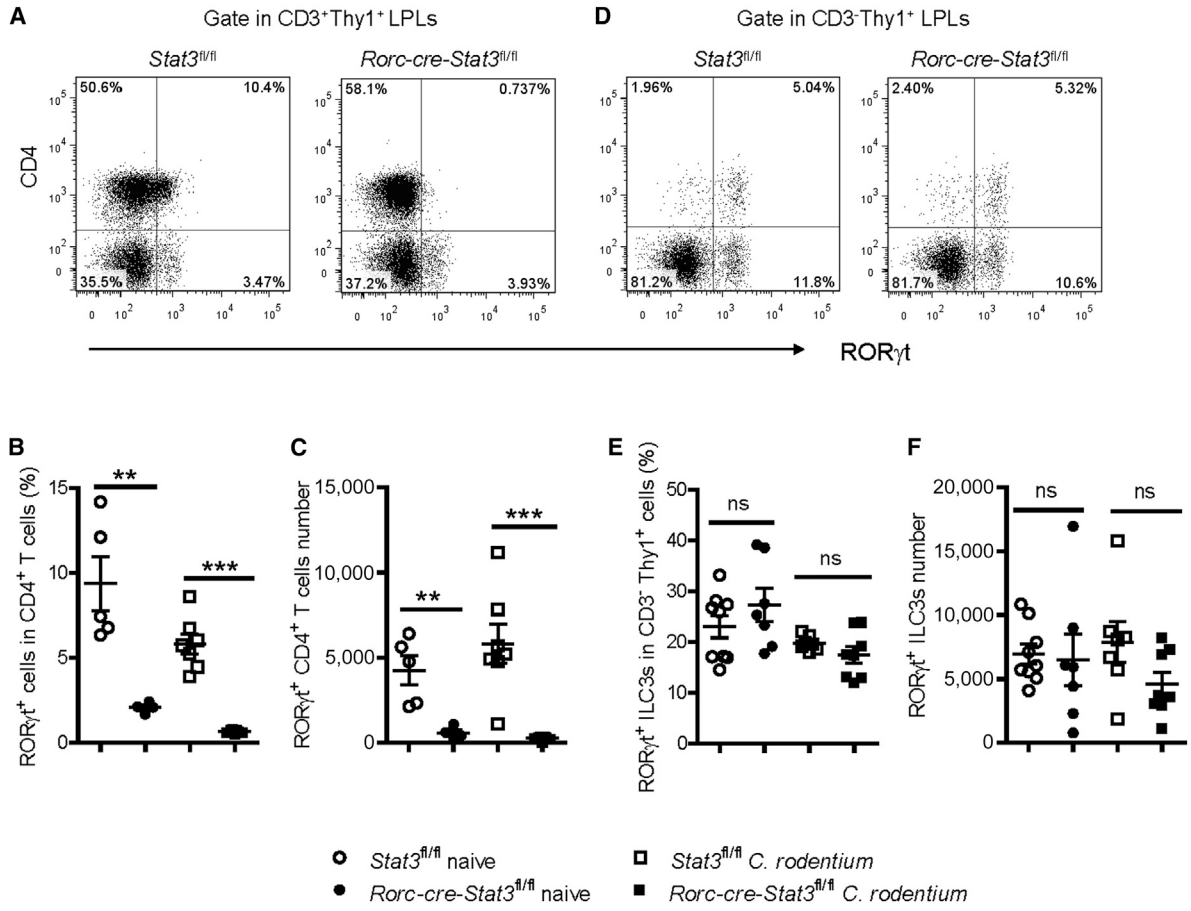

Figure 6. The Development of RORγt+ T Helper Cells but Not RORγt+ ILCs Is Dependent on STAT3 Signaling

Colonic LPLs were isolated from *Stat3*$^{fl/fl}$ and *Rorc-cre-Stat3*$^{fl/fl}$ littermate mice before or 5 days after *C. rodentium* infection.

(A and D) RORγt and CD4 expression were analyzed in colonic LPLs from naive mice by flow cytometry after gating on Thy1$^+$CD3$^+$ adaptive lymphocytes and Thy1$^+$CD3$^-$ innate lymphoid cells.

(B and C) Percentage of RORγt$^+$ cells in the CD4$^+$CD3$^+$ T cell population as well as the absolute numbers of RORγt$^+$CD4$^+$ T cells in the colons of naive and *C. rodentium*-infected *Stat3*$^{fl/fl}$ and *Rorc-cre-Stat3*$^{fl/fl}$ mice are shown.

(E and F) Percentage of RORγt$^+$ cells in the CD3$^-$Thy1$^+$ ILCs population as well as the absolute numbers of RORγt$^+$ ILCs in the colons of naive and *C. rodentium*-infected *Stat3*$^{fl/fl}$ and *Rorc-cre-Stat3*$^{fl/fl}$ mice are shown.

$^{**}p < 0.01$, $^{***}p < 0.001$; ns, no significant difference (Student's t test). Data are representative of three independent experiments (mean ± SEM). Each dot represents one individual mouse (B, C, E, F). See also Figure S6.

Together, our studies have demonstrated several important findings. (1) In contrast to the essential role of controlling the expression of RORγt in T cells, STAT3 has little impact on the development of RORγt$^+$ ILC3s, but rather directly regulates the function of innate RORγt$^+$ ILC3s, which is essential for gut immunity. (2) Although STAT3 regulates the function of both innate and adaptive RORγt$^+$ cells, our data suggest that STAT3 signaling and IL-22 from innate RORγt$^+$ ILC3s but not from adaptive T cells are essential in the response to acute mucosal bacterial infections. (3) Previous studies have shown that STAT3 signaling controls IL-22 production by T cells through regulating RORγt expression. Our data show that STAT3 can directly control IL-22 production. (4) STAT3 inhibition in a major antitumor therapy also impairs the host protection from intestinal bacterial infection. Exogenous IL-22 administration may be beneficial for reducing side effects in the cancer patients undergoing STAT3 inhibitor treatment.

(B–D) *Rag1*$^{-/-}$ mice were infected with 2 × 10^9 CFU of *C. rodentium* and CD90^{hi}CD45^{lo} ILC3s were sorted from both the colon and small intestine LPLs at day 1 postinfection. Sorted ILC3s were stimulated with IL-23 (25 ng/ml) for 1 hr, then transferred by i.v. injection (2 × 10^5 cells per mouse) into *Rorc-cre-Stat3*$^{fl/fl}$ recipients at day 0 and day 3 after inoculation with 5 × 10^6 CFU of *C. rodentium*. Untreated *Rorc-cre-Stat3*$^{fl/fl}$ and wild-type *Stat3*$^{fl/fl}$ littermate mice were controls.

(B and C) Body weight changes (B) and survival rates (C) were monitored at indicated time points.

(D) CFU counts of *C. rodentium* in the fecal pellets and blood on day 5 after infection are shown. Data are pooled from two independent experiments with 9–11 mice per group (mean ± SEM). $^{***}p < 0.001$ (Student's t test).

See also Figure S5.

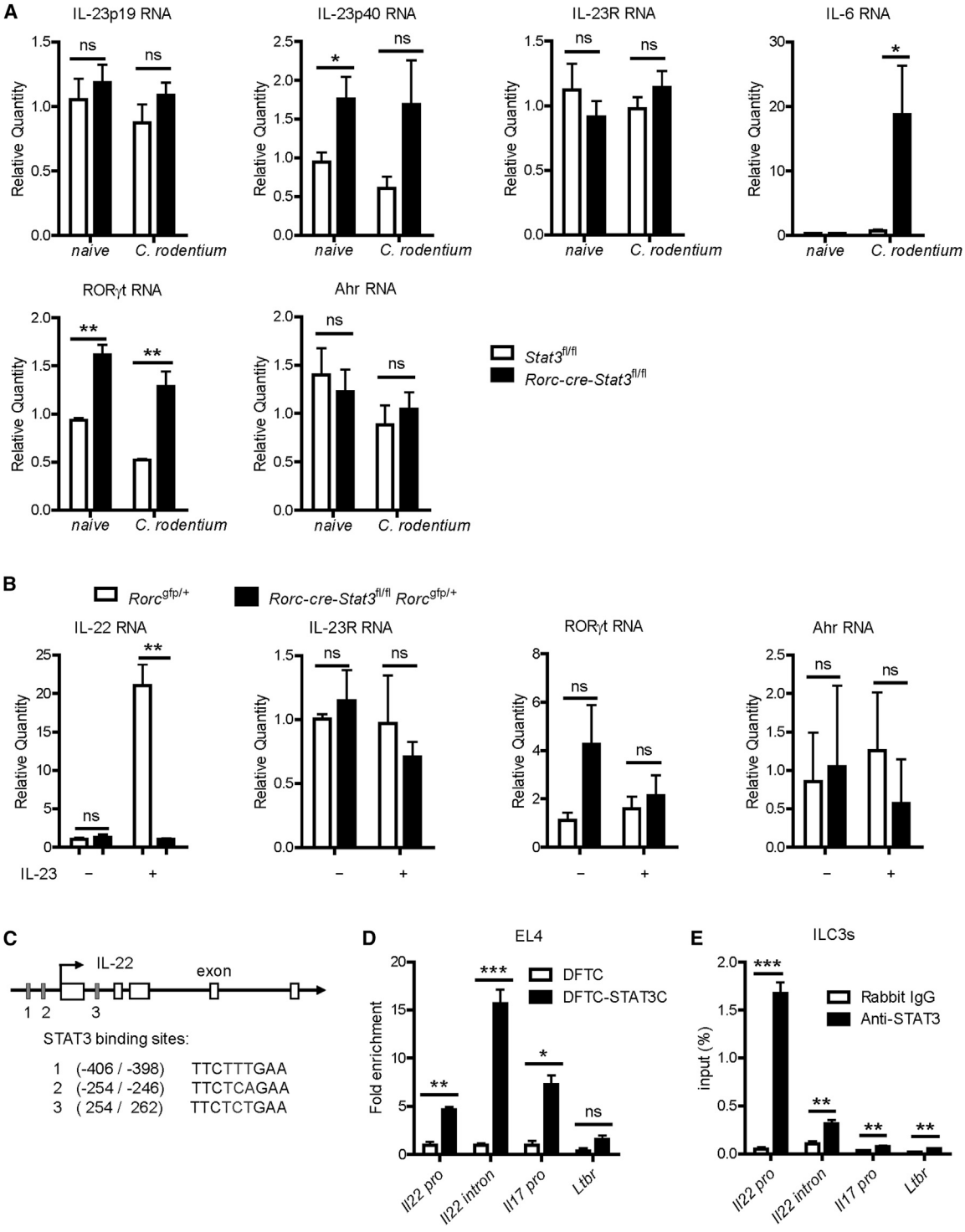

Figure 7. STAT3 Directly Binds to the *Il22* Locus and Regulates IL-22 Production

(A) *Rorc-cre-Stat3*$^{fl/fl}$ and *Stat3*$^{fl/fl}$ littermate mice (n = 5 per group) were infected with 2 × 10^9 CFU of *C. rodentium*. The mRNA expression of IL-23 p19, IL-23 p40, IL-23R, IL-6, RORγt, and Ahr in the colon were measured by real-time PCR before and 5 days after infection. Data are representative of two independent experiments (mean ± SEM).

(B) RORγt$^+$ ILCs were purified by flow cytometric sorting from colonic LPLs of *Rorc-cre-Stat3*$^{fl/fl}$*Rorc*$^{gfp/+}$ and *Rorc*$^{gfp/+}$ mice and stimulated with or without IL-23 (25 ng/ml) for 1 hr. The mRNA expression of IL-22, IL-23R, RORγt, and Ahr were measured by real-time PCR. Data are representative of two independent experiments (mean ± SEM).

(C) STAT3 binding sites at the *Il22* locus are shown.

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EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and *Rag1*$^{-/-}$ mice were purchased from Harland Teklad. *Cd4-cre* (Lee et al., 2001) mice were purchased from Taconic. *Rorc*$^{gfp/+}$ (Eberl et al., 2004) mice were purchased from The Jackson Lab. *Rorc-cre-Stat3*$^{fl/fl}$, *Rorc-cre-Stat3*$^{fl/+}$, and *Cd4-cre-Stat3*$^{fl/fl}$ mice were generated by crossing *Stat3*-floxed mice (Takeda et al., 1998) with *Rorc-cre* (Eberl and Littman, 2004) and *Cd4-cre* transgenic mice, respectively. *Rorc-cre-Stat3*$^{fl/fl}$*Rorc*$^{gfp/+}$ mice were generated by crossing *Rorc-cre-Stat3*$^{fl/fl}$ mice with *Rorc*$^{gfp/+}$ mice. All mice are on C57BL/6 background. Animal care and use were in accordance with institutional and National Institutes of Health guidelines and all studies were approved by the Animal Care and Use Committee of the University of Chicago and Northwestern University.

Infection with *C. rodentium* and Treatment

Mice were orally gavaged with *C. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection) and body weight, survival, colony-forming units (CFUs) counts, and tissue histology were assessed as previously described (Tumanov et al., 2011; Wang et al., 2010). Colon culture was done as previously described (Zheng et al., 2008). Where indicated, Sunitinib (SU11248; SUTENT) or control was gavaged orally, once a day, at doses of 4 or 40 mg/kg body weight after infection. Where indicated, mice were injected intraperitoneally with mouse IL-22-Ig fusion protein on 0, 2, and 4 days after *C. rodentium* infection at a dose of 50 μg per mouse each time. Control groups received isotype control immunoglobulin. For depletion of CD4$^+$ T lymphocytes, mice were injected intraperitoneally with mAb GK1.5 or rat IgG on −5, 0, 5, 10, and 15 days after *C. rodentium* infection at a dose of 50 μg per mouse each time. Where indicated, IL-17- and IL-22-expressing plasmids were introduced into mice by a hydrodynamic tail vein injection-based gene transfer technique (Tumanov et al., 2011).

Isolation of Intestinal LPLs

The isolation of intestinal lamina propria cells was done as previously described (Wang et al., 2010). In brief, mice were killed and colons were removed and placed in ice-cold PBS. The intestines were cut open longitudinally, thoroughly washed in ice-cold PBS, and cut into 1.5 cm pieces. Intestines were incubated with shaking in RPMI 1640 (Invitrogen) containing 3% FBS, 1 mM DTT, 5 mM EDTA, and 10 mM HEPES at 37°C for 30 min. After washing with RPMI 1640 containing 2 mM EDTA, the tissues were then digested in RPMI 1640 containing 0.05% DNase I (Sigma) and Liberase (Roche) (0.1 mg/ml) at 37°C for 30 min with slow rotation. The digested tissues were homogenized by vigorous shaking and passed through 100 μm cell strainer. Mononuclear cells were then harvested from the interphase of an 80% and 40% Percoll gradient after a spin at 2,500 rpm for 20 min at room temperature.

Flow Cytometry, Antibodies, and ELISA

Antibodies against CD3, CD4, CD8, CD45, CD90, NKp46, Gr-1, CD11b, KLRG1, TCRβ, NK1.1, and Streptavidin-APC were purchased from BioLegend. Antibodies against RORγt and IL-17 were purchased from eBioscience. Antibodies against STAT3 and rabbit IgG were purchased from Cell Signaling Technology. Anti-IL-22 antibody was a gift from Genentech. For nuclear staining, cells were fixed and permeabilized with a Transcription Factor Staining Buffer Set (eBioscience). For cytokine production, cells were stimulated ex vivo by IL-23 (25 ng/ml, R&D), PMA (50 ng/ml, Sigma) and ionomycin (750 ng/ml, Calbiochem), or anti-CD3 (plate coated, 1 μg/ml) and anti-CD28 (2 μg/ml) for 4 hr and Brefeldin A (10 μg/ml, eBioscience) was added 2 hr before cells were harvested for analysis. IC Fixation Buffer and Permeabilization Buffer were used according to the manufacturer's instructions (eBioscience) for intracellular cytokine staining. Flow cytometry analysis was performed on FACSCanto (BD Biosciences) instruments and analyzed with FlowJo software (Tree Star). IL-22 (R&D Systems), IL-17, and IL-23 (eBioscience) in supernatants were measured by ELISA according to the manufacturer's recommendations.

Quantitative Real-Time RT-PCR

RNA from tissues was isolated with Trizol reagent (Invitrogen) and reverse transcribed with AMV Reverse Transcriptase (Promega). RNA from sorted cells was isolated with the RNeasy Micro Kit (QIAGEN) and was reverse transcribed with Seniscript Reverse Transcription Kit (QIAGEN). Real-time RT-PCR was performed with SSoFast EvaGreen supermix (Bio-Rad) and different primer sets (Table S1) on StepOne Plus (Applied Biosystems). Samples were normalized to HPRT and reported according to the $2^{-\Delta\Delta CT}$ method.

Adoptive Transfer Experiments

Rag1$^{-/-}$ mice were infected with *C. rodentium*. CD90^{hi}CD45^{lo} ILC3s were sorted from both the colon and small intestine LPLs on a FACS Aria III instrument (BD Bioscience) at day 1 postinfection. Sorted ILC3s were stimulated with IL-23 (25 ng/ml) for 1 hr, then were transferred by i.v. injection (2×10^5 cells per mouse) into recipients inoculated with *C. rodentium* at day 0 and day 3.

Chromatin Immunoprecipitation

ChIP assays with EL4 cells were performed as previously described (Qiu et al., 2012). In brief, 5×10^7 EL4 cells were first fixed in 1% formaldehyde in RPMI 1640 media for 10 min at room temperature. The reaction was then stopped by adding glycine solution. Fixed cells were washed with PBS and resuspended in lysis buffer. Chromatin was sheared by sonication on ice, and the insoluble fraction was removed by centrifugation. The lysate was precleared with Protein A beads (Santa Cruz Biotechnology) and then incubated with anti-Flag beads (Sigma) at 4°C for 5 hr. After washing, precipitated chromatin fragments were eluted with flag peptide (Sigma). The samples were then reverse cross-linked at 65°C by adding Tris-EDTA with 1% SDS. After proteinase K digestion, DNA was extracted with Phenol-Chloroform and precipitated for quantitative real-time PCR analyses by specific primers. CD90^{hi}CD45^{lo} ILC3s were sorted from *Rag1*$^{-/-}$ mice and ChIP assays were performed with anti-STAT3 rabbit mAb (D3Z2G) or rabbit IgG (Cell Signaling Technology). MegnaChIP protein A magnetic beads (Millipore) were used to isolate immune complexes. Cross-links were reversed by heating at 65°C and proteinase K treatment. DNA was purified with Qiaquick PCR purification kit (QIAGEN).

Statistical Methods

Statistical analysis was performed by two-tailed Student's t test on GraphPad Prism 5.0 program. Data from such experiments are presented as mean values ± SEM; $p < 0.05$ was considered significant. For survival curves, statistics were done with the log rank (Mantel-Cox) test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.10.021.

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(D) STAT3 binding at the *Il22* locus in EL4 cell lines stably expressing either flag peptide (DFTC) or flag-tagged STAT3C (DFTC-STAT3C) was monitored by a ChIP assay. The anti-flag antibody immunoprecipitates were analyzed by real-time PCR. The fold enrichment of STAT3C binding at each locus was normalized to DFTC-empty EL4 cells. *Ltbr* and *Il17* loci were used as negative and positive controls in the ChIP assays, respectively. Data are representative of three independent experiments (mean ± SEM of triplicate samples of real-time PCR).
(E) CD90^{hi}CD45^{lo} ILC3s were sorted from *Rag1*$^{-/-}$ mice and stimulated with IL-23 (25 ng/ml) for 30 min. ChIP assays with anti-STAT3 mAb showed enhanced enrichment of STAT3 at the *Il22* locus in primary ILC3s. *Ltbr* loci lacking STAT3 binding sites was used as negative controls. Data are representative of two independent experiments (mean ± SEM of quadruplicate samples of real-time PCR).
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significant difference (Student's t test).

(New York University, NY) for *Rorc-cre* mice; W. Ouyang (Genentech, CA) for IL-22-expressing plasmid, IL-22-Ig and anti-IL-22 antibody; C. Dong (MD Anderson Cancer Center, TX) for IL-17 plasmid; V. Krishnamoorthy (University of Chicago, IL) for ChIP protocol; and S. Kron and J. Cunningham (University of Chicago, IL) for sharing sonicator. The work was supported by the National Institutes of Health grants (AI 089954 and AI 091962 to L.Z., DK080736 and CA141975 to Y.X.F.), by a Pew Scholarship and by a Cancer Research Institute Investigator Award (to L.Z). L.Z. is a BWF Investigator in the Pathogenesis of Infectious Disease.

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